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Analysis of ATP-citrate lyase and malic enzyme mutants of *Yarrowia lipolytica* points out the importance of mannitol metabolism in fatty acid synthesis

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ABSTRACT

The role of the two key enzymes of fatty acid (FA) synthesis, ATP-citrate lyase (Acl) and malic enzyme (Mae), was analyzed in the oleaginous yeast *Yarrowia lipolytica*. In most oleaginous yeasts, Acl and Mae are proposed to provide, respectively, acetyl-CoA and NADPH for FA synthesis. Acl was mainly studied at the biochemical level but no strain depleted for this enzyme was analyzed in oleaginous microorganisms. On the other hand the role of Mae in FA synthesis in *Y. lipolytica* remains unclear since it was proposed to be a mitochondrial NAD(H)-dependent enzyme and not a cytosolic NADP(H)-dependent enzyme. In this study, we analyzed for the first time strains inactivated for corresponding genes. Inactivation of *ACL1* decreases FA synthesis by 60 to 80%, confirming its essential role in FA synthesis in *Y. lipolytica*. Conversely, inactivation of *MAE1* has no effects on FA synthesis, except in a FA overaccumulating strain where it improves FA synthesis by 35%. This result definitively excludes Mae as a major key enzyme for FA synthesis in *Y. lipolytica*. During the analysis of both mutants, we observed a negative correlation between FA and mannitol level. As mannitol and FA pathways may compete for carbon storage, we inactivated *YlSDR*, encoding a mannitol dehydrogenase converting fructose and NADPH into mannitol and $NADP^+$. The FA content of the resulting mutant was improved by 60% during growth on fructose, demonstrating that mannitol metabolism may modulate FA synthesis in *Y. lipolytica*.

1. Introduction

Utilization of microbial oils as a substrate for biofuel production represents a promising way to face global change and rarefaction of petroleum resources. Few micro-organisms are able to produce a high level of oil [1,2]. Among them, yeasts have emerged as good candidates [2,3], because they are easy to cultivate, to manipulate genetically and they have a high lipid accumulation potential. For this reason, improvement of fatty acid (FA) accumulation in yeasts has become a very important topic in recent years [4–11] and will be probably still of high importance in the next years.

FA is produced by the Fatty Acid Synthase (FAS) from acetyl-CoA, malonyl-CoA and NADPH. For several decades, provision of acetyl-CoA and NADPH has been attributed to ATP-citrate lyase (Acl) and malic enzyme (Mae), respectively [12,13]. However, the role of these two major enzymes appears not so clear, depending in which organism is being studied.

Acl converts cytosolic citrate, CoA and ATP into acetyl-CoA, ADP + Pi and oxaloacetate (Fig. 1). This cytosolic enzyme is present in few *Prokaryotes* and in all *Eukaryotes*, but not in non-oleaginous yeasts [14]. Thus, this enzyme was presumed to be essential for FA synthesis [1,13,15]. In most microorganisms and in plants, Acl is composed of two subunits, encoded by the *ACL1* and *ACL2* genes [16], while in animals and in the oleaginous *Basidiomycete* yeast, *Rhodotorula gracilis*, Acl is encoded by one gene [17–19]. In the non-oleaginous fungus *Aspergillus niger*, inactivation of *ACL1* and/or *ACL2*, results in a decrease of acetyl-CoA and citric acid levels, vegetative growth, pigmentation, asexual conidiogenesis, and conidial germination and an increase of succinic acid level [20,21]. Unfortunately, the effect of Acl inactivation on FA synthesis was not described in this fungus. Additionally, overexpression of *ACL1* and *ACL2* generated variable results depending on the organism. In *Aspergillus oryzae* it leads to an 1.7-fold increase of FA content [22] whereas no effect was observed when these genes were overexpressed in the wild type (WT) or in citrate overproducing strains

Abbreviations: CDW, Cell Dry Weight; FA, Fatty Acid; FFA, Free Fatty Acid; LB(s), Lipid Body(ies); TAG, Triglycerides; TGL, Intracellular Triglycerides Lipase

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of *Saccharomyces cerevisiae*, or in WT strain of *Yarrowia lipolytica* [9,10]. Few studies have examined Acl in oleaginous microorganisms. Recently, Liu et al. [23] and Ochoa-Estopier and Guillouet [24] have shown that high activity of Acl is usually observed during the FA synthesis phase in *Trichosporon cutaneum* and in *Y. lipolytica*. However, no mutant inactivated for Acl has ever been described in an oleaginous organism.

Mae is a cytosolic enzyme involved in conversion of L-malate and $NADP^+$ into pyruvate, CO_2 and NADPH (Fig. 1) [25]. NADPH is essential for FA synthesis and due to its capacity to produce NADPH, this enzyme was proposed to be one of the rate-limiting steps for FA synthesis [12,26]. In oleaginous microorganisms, the role of Mae was only deduced from its activity during lipid accumulation [23,24,27] or from overexpression studies, never in strains inactivated for the corresponding gene. As an example, overexpression of Mae from *Mucor circinelloides* improved lipid accumulation by 2.5- and 2-fold in *M. circinelloides* and in the oleaginous *Basidiomycetes* yeast *Rhodotorula glutinis*, respectively [28,29]. The oleaginous yeast, *Y. lipolytica*, does not possess cytosolic Mae, but only a mitochondrial form, which may not be involved in NADPH production [30]. The role of this mitochondrial NADH-dependent Mae remains unclear. Moreover the overexpression of cytosolic NADPH-dependent Mae from *Mortierella alpina* failed to improve lipid synthesis in *Y. lipolytica*, raising the question of NADPH supply in this yeast. The question of provision of NADPH for FA synthesis in *Y. lipolytica* became a "hot topic" since Wasylenko et al. [31] showed that pentose phosphate pathway is the primary source of lipogenic NADPH in *Y. lipolytica* and C. Ratledge [25] just proposed alternative routes to pentose phosphate pathway to provide NADPH to FA synthesis.

In *Ascomycetes* fungi, the mannitol cycle was proposed to be a provider of reducing power [32]. This cycle consists of two pathways (Fig. 4). The first pathway involves in the reduction of fructose-6-phosphate, from glycolysis, into mannitol-1-phosphate, by the NAD(H)-dependent mannitol-1-phosphate dehydrogenase (Mpd), which is then dephosphorylated into mannitol via a mannitol-1-phosphate phosphatase. This last reaction was described as irreversible, but some data suggest that it is not the case in all fungi [33]. In the second pathway, fructose is oxidized into mannitol via a reversible NADP(H)-dependent mannitol 2-dehydrogenase (Mdh). One turn of this cycle gives the net result: NADH + $NADP^+$ + ATP converted to NAD^+ + NADPH + ADP + Pi, i.e. the same net result as the reactions needed for NADPH production from malate with the cytosolic Mae [25]. As *Y. lipolytica* is known to produce mannitol [34], this raises the question of a potential role of the mannitol metabolism in FA synthesis in this yeast.

In the present study we sought to decipher the role of ATP-citrate lyase and of the mitochondrial NADH-dependent Mae in the oleaginous yeast *Y. lipolytica*. Indeed, although this yeast is one of the major models for lipid metabolism, the function of these enzymes is still not clear. As the study of these genes has revealed the existence of a link between mannitol and FA, the interconnection between both metabolisms was analyzed.

2. Material and methods

2.1. Yeast strains, growth, and culture conditions

The *Y. lipolytica* strains used in this study were derived from the wild-type (WT) *Y. lipolytica* W29 (ATCC20460) strain (Table 1). The auxotrophic strain, PO1d (Leu$^-$ Ura$^-$) has been previously described by Barth and Gaillardin [35]. The high FA acid producing prototrophic strain JMY3501 and its auxotrophic derivative strain, JMY3820, have been previously described by Lazar et al. [36]. We rendered all the strains prototrophic to avoid growth defects due to leucine auxotrophy, as reported previously by Mauersberger et al. [37]. Strains recovering the wild-type *LEU2* locus, after transformation with a *Sal*I fragment

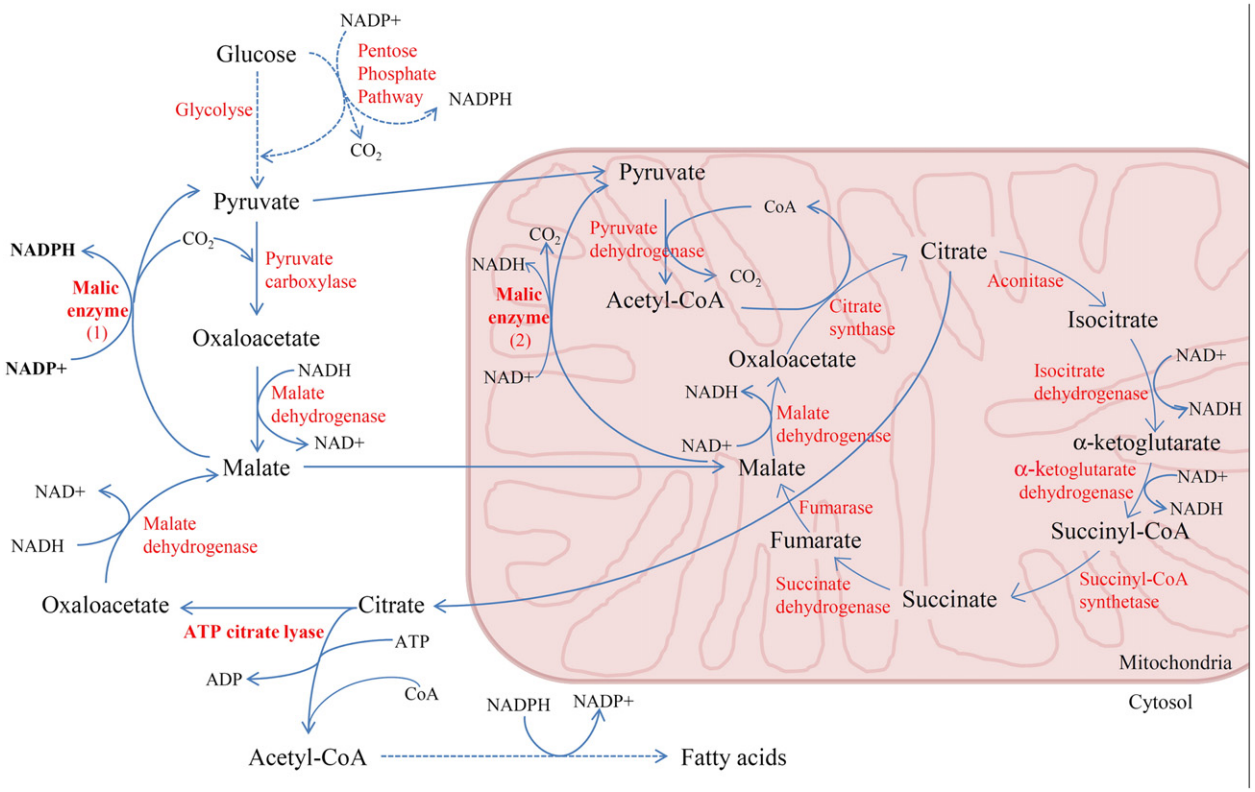

Fig. 1. General pathways for the production of FA in oleaginous fungi. Main cytosolic and mitochondrial enzymes and pathways (in red) required for utilization of glucose and production of main molecules necessary for FA synthesis (NADPH, citrate, acetyl-CoA). Malic enzyme was described as cytosolic (1) and/or mitochondrial (2) enzymes, depending on the organism. Dashed arrows indicate a series of enzymatic reactions.

Table 1
Strains used in this study.

Strain (host strain)	Genotype or plasmid	Source or reference
E. coli		
Mach1T1	*ΔrecA1398 endA1 tonA Φ80ΔlacM15 ΔlacX74 hsdR(rk − mk+)*	Invitrogen
pINA62	*Y. lipolytica LEU2* in pBR322	[38]
JME547	pUB4-*CRE 1*	[43]
JME1128	JMP62 *URA Ex-pTEF-GPD1*	[5]
JME982	pKS *ACL1-PT*	This work
JME991	pKS *ACL1-PLT* cassette	This work
JME1619	JMP62 *URA3ex pTEF-ACL1*	Verbeke J. unpublished data
JMY1855	JMP62 *URA3ex pTEF-ACL2*	Verbeke J. unpublished data
JMY2246	JMP62 *LEU2ex pTEF-ACL2*	This work
JMY2372	TOPO *MAE1-PT*	This work
JMY2374	TOPO *MAE1-PLT* cassette	This work
JMY2479	TOPO *YlSDR-PT*	This work
JMY2495	TOPO *YlSDR-PLT*	This work
JMY2509	TOPO *YlSDR-PUT*	This work
JMY2550	JMP62 *URA3ex pTEF-YlSDR*	This work
Y. lipolytica		
W29	*MAT*a WT	[35]
PO1d	*MAT*a *ura3-302 leu2-270 xpr2-322*	[35]
JMY330	*MAT*a *ura3-302 leu2-270 xpr2-322 URA3ex*	[36]
JMY2900	*MAT*a *ura3-302 xpr2-322 URA3ex* (called "**Reference strain**")	[36]
JMY3501	*MAT*a *ura3-302 leu2-270 xpr2-322 Δpox1-6 Δtgl4 + pTEF-DGA2 LEU2ex + pTEF-GPD1 URA3ex* (called "**Obese**")	[36]
JMY3820	*MAT*a *ura3-302 leu2-270 xpr2-322 Δpox1-6 Δtgl4 + pTEF-DGA2 + pTEF-GPD1*	[36]
JMY1580	*MAT*a *ura3-302 leu2-270 xpr2-322 acl1::URA3ex*	This work
JMY2215	*MAT*a *ura3-302 xpr2-322 acl1::URA3ex* (called "***Δacl1***")	This work
JMY4146	*MAT*a *ura3-302 leu2-270 xpr2-322 + pTEF-ACL2-LEU2ex*	This work
JMY4175	*MAT*a *ura3-302 leu2-270 xpr2-322 + pTEF-ACL1-URA3ex + pTEF-ACL2-LEU2ex*	This work
JMY3904	*MAT*a *ura3-302 leu2-270 xpr2-322 Δpox1-6 Δtgl4 + pTEF-DGA2 + pTEF-GPD1 + pTEF-ACL1-URA3ex*	This work
JMY4079	*MAT*a *ura3-302 leu2-270 xpr2-322 Δpox1-6 Δtgl4 + pTEF-DGA2 + pTEF-GPD1 + pTEF-ACL1-URA3ex + pTEF-ACL2-LEU2ex* (called "**Obese + *ACL1/2***")	This work
JMY4162	*MAT*a *ura3-302 leu2-270 xpr2-322 mae1::LEU2ex URA3ex* (called "***Δmae1***")	This work
JMY4300	*MAT*a *ura3-302 leu2-270 xpr2-322 Δpox1-6 Δtgl4 mae1::LEU2ex + pTEF-DGA2 + pTEF-GPD1*	This work
JMY4501	*MAT*a *ura3-302 leu2-270 xpr2-322 Δpox1-6 Δtgl4 mae1::LEU2ex + pTEF-DGA2 + pTEF-GPD1 URA3ex* (called "**Obese *Δmae1***")	This work
JMY4381	*MAT*a *ura3-302 leu2-270 xpr2-322 Ylsdr::LEU2ex URA3ex* (called "***ΔYlsdr***")	This work
JMY4450	*MAT*a *ura3-302 leu2-270 xpr2-322 Δpox1-6 Δtgl4 Ylsdr::URA3ex + pTEF-DGA2 + pTEF-GPD1*	This work
JMY4484	*MAT*a *ura3-302 leu2-270 xpr2-322 Δpox1-6 Δtgl4 Ylsdr::URA3ex + pTEF-DGA2 + pTEF-GPD1 + LEU2* (called "***Obese ΔYlsdr***")	This work
JMY4808	*MAT*a *ura3-302 leu2-270 xpr2-322 + pTEF-YlSDR URA3ex*	This work
JMY4812	*MAT*a *ura3-302 leu2-270 xpr2-322 + pTEF-YlSDR URA3ex LEU2ex* (called "***+ YlSDR***")	This work
JMY4451	*MAT*a *ura3-302 leu2-270 xpr2-322 Δpox1-6 Δtgl4 + pTEF-DGA2 + pTEF-GPD1 + pTEF-YlSDR URA3ex*	This work
JMY4487	*MAT*a *ura3-302 leu2-270 xpr2-322 Δpox1-6 Δtgl4 + pTEF-DGA2 + pTEF-GPD1 + pTEF-YlSDR URA3ex + LEU2* (called "**Obese + *YlSDR***")	This work
JMY5111	*MAT*a *ura3-302 leu2-270 xpr2-322 Δpox1-6 Δtgl4 Ylsdr::URA3ex mae1::LEU2ex + pTEF-DGA2 + pTEF-GPD1* (called "***Obese Δmae1ΔYlsdr***")	This work

from pINA62 carrying the *LEU2* gene [38], were selected and used for further analysis. All the strains used in this study are listed in Table 1.

Media and growth conditions for *Escherichia coli* have been previously described by Sambrook et al. [39], and those for *Y. lipolytica* have been described by Barth and Gaillardin [35]. Rich medium (YPD) and minimal glucose medium (YNB) were prepared as described previously [40]. Minimal medium $YNBD_2$ medium, called in the text non-lipogenic medium, contained 0.17% (wt/vol) yeast nitrogen base (without amino acids and ammonium sulfate, YNBw/o; Difco, Paris, France), 2% glucose (wt/vol; Merck, Fontenay-sous-Bois Cedex, France), 0.5% (wt/vol) NH_4Cl and 50 mM phosphate buffer (pH 6.8). To complement auxotrophy, 0.01% uracil or 0.01% leucine is added to $YNBD_2$. The YNBCN30 medium, called in the text lipogenic medium, used for optimal de novo FA synthesis contained 0.17% (wt/vol) yeast nitrogen base (YNBww), 3% glucose (wt/vol; Merck, Fontenay-sous-Bois Cedex, France), 0.15% (wt/vol) NH_4Cl and 50 mM phosphate buffer (pH 6.8).

Medium optimized for mannitol production consisted of 0.27% $(NH_4)_2SO_4$, 0.02% KH_2PO_4, 0.16% yeast extract, 0.1% $MgSO_4 \times 7H_2O$, and 0.3% $CaCO_3$. The pH was adjusted to 3 and the medium was supplemented with 10% glycerol (Mgly10%) or 10% fructose (Mfru10%) (Juszczyk P. personal communication).

Cultivation was typically performed as follows. From a YPD plate, an initial preculture was inoculated into YPD medium (160 rpm, 28 °C, overnight). For the experimental culture, cells from the overnight preculture were harvested by centrifugation, washed, and resuspended in fresh medium to an optical density (A_{600}) of 0.5.

2.2. General genetic techniques

Standard molecular genetic techniques were used throughout this study [39]. Restriction enzymes were obtained from OZYME (Saint-Quentin-en-Yvelines, France). Genomic DNA from yeast transformants was prepared as described by Querol et al. [41]. PCR amplifications were performed in an Applied Biosystems 2720 thermal cycler with Go Taq (Promega, Charbonnieres, France) or Pyrobest (Takara, Saint-Germain-en-Laye, France) DNA polymerases. ORFs from *ACL1*, *ACL2*, *MAE1* and promoter (P) and terminator (T) regions from *ACL1*, *MAE1* and *YlSDR* were amplified by PCR using the primers shown in Table S1. PCR fragments were purified with a QIAgen Purification Kit (Qiagen, Hilden, Germany), and DNA fragments were recovered from agarose gels with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The Staden software package was used for gene sequence analysis [42].

2.3. Construction of plasmids and Y. lipolytica mutant strains

The deletion cassettes were typically generated by PCR amplification according to Fickers et al. [43]. First, the promoter (P) and terminator (T) regions were amplified from *Y. lipolytica* W29 genomic DNA as the template and with the gene-specific P1/P2 and T1/T2 oligonucleotides as primer pairs (Table S1). Primers P2 and T1 contained an extension to introduce the *I-Sce*I restriction site, whereas P1 and T2 contained an extension to introduce the *Not*I restriction site.

For the *ACL1* gene, primer pairs ACL1-P1/ACL1-P2 and ACL1-T1/ACL1-T2 were used (Table S1). The P and T regions were purified and used for the second PCR. The resulting PT fragment was ligated into pKS, yielding the construct JME982. The *URA3* marker was then introduced at the *I-Sce*I site, yielding the construct JME991 containing the *ACL1-PUT* cassette. PCR fragment, corresponding to PUT cassette, was amplified using ACL1-P1 and ACL1-T2 and was transformed into PO1d strain to obtain mutants inactivated for *ACL1*.

For the *MAE1* and *YlSDR* genes, primer pairs *MAE1*-P1/*MAE1*-P2 and *MAE1*-T1/*MAE1*-T2, and *YlSDR*-P1/*YlSDR*-P2 and *YlSDR*-T1/*YlSDR*-T2 were used, respectively (Table S1). The P and T regions were purified and fused using corresponding P1 and T2 primers. The resulting PT fragment was inserted into pCR4Blunt-TOPO, yielding the construct JME2372 and JME2479. A marker, *LEU2ex*, was then introduced at the *I-Sce*I site, yielding the constructs JME2374 and JME2495 containing the *MAE1-PLT* and the *YlSDR-PLT* cassettes, respectively. *MAE1-PLT* and *YlSDR-PLT* cassettes were recovered by a *Not*I digestion and transformed into JMY330 and JMY3820 to obtain mutants inactivated for *MAE1* or for *YlSDR* (Table 1).

The disruption cassettes (PUT and PLT) or the *LEU2* fragment (from pINA62) were used for transformation by the lithium acetate method [44]. Transformants were selected on YNB supplemented with leucine or uracil for Ura^+ Leu^- and Ura^- Leu^+ strains, respectively, and on YNB for Ura^+ Leu^+ strains. Transformants were analyzed by PCR with ver1/ver2 primer pairs (Table S1).

The overexpression cassettes were generated as follow. ORFs of *ACL1* (*YALI0E34793g*), *ACL2* (*YALI0D24431g*), *MAE1* (*YALI0E18634g*) and *YlSDR* (*YALI0B16192g*), were amplified using primers presented in Table S1. CDS-F and CDS-R primers contained an extension to introduce the *BamH*I or *Avr*II restriction sites, respectively. As *ACL2* CDS sequence possesses 2 *BamH*I restriction sites, it was necessary to modify these sites by fusion PCR (Table S1), to avoid modifying final protein sequence. JMP1128 was digested by *BamH*I and *Avr*II to replace expressing *GPD1* gene by ORF from *MAE1*, *ACL1*, *ACL2* or *YlSDR* to generate JME1619, JME1855 and JME2248 and JME2550, respectively. JME2246 was obtained by replacing the *URA3ex* selective marker from JME1855 by the *LEU2ex* cassette (*I-Sce*I digestion). *GPD1*- (from JME1128 [5]), *DGA2*- (from JME1822 [4]), *ACL1*- (from JME1619), *ACL2*- (from JME2246), *MAE1*- (from JME2248), and *YlSDR*- (from JME2550) overexpression cassettes, were recovered by *Not*I digestion and introduced into different *Y. lipolytica* strains (Table 1). Transformants were analyzed by PCR with pTEF-START primer and the CDS-R primers used for CDS amplification (Table S1).

Auxotrophies were restored by excision using the Cre-lox recombinase system upon transformation with the replicative plasmid pUB4-*CRE1* (JME547) as described by Fickers et al. [43].

2.4. Fluorescence microscopy

For visualization of lipid bodies (LBs), BodiPy® lipid probe (2.5 mg/ml in ethanol; Life Technologies, Saint Aubin, France) was added to the cell suspension (A_{600} of 10) and incubated for 5 min at room temperature. Microscopy was performed with a fluorescence microscope, AXIO Imager M2 (Zeiss, Le Pecq, France) at 495 nm with a 100× oil-immersion objective. AxioVision Rel. 4.6 software was used for recording the images.

2.5. Lipid determination

Lipids from an equivalent weight of cells (20 mg) were used for gas chromatography (GC) analysis after being converted into their methyl esters with freeze-dried cells according to the procedure Browse [44]. GC analysis of FA methyl esters was performed with a Varian 3900 instrument equipped with a flame-ionization detector and a Varian FactorFour vf-23 ms column, where the bleed specification at 260 °C is 3 pA (30 m, 0.25 mm, 0.25 μm). FA was identified by comparison with commercial FA methyl ester standards (FAME32; Supelco) and was quantified by the internal standard method of adding 100 μg of commercial C12:0 (Sigma-Aldrich).

2.6. Sugar, polyol and organic acid quantification

Glucose, mannitol, fructose, glycerol, citrate and malate were identified and quantified by HPLC (UltiMate 3000, Dionex-Thermo Fisher Scientific, UK) using an Aminex HPX87H column or a HyperRez Carbohydrate H^+ Column (Thermo Scientific, Waltham, MA) coupled to UV (210 nm) and RI detectors. The Aminex HPX87H column was eluted with 0.01 N H_2SO_4 at 35 °C temperature and a flow rate of 0.6 mL/min. The HyperRez Carbohydrate H^+ Column was eluted with 25 mM trifluoroacetic acid (TFA) at 65 °C and a flow rate of 0.6 mL/min. Peak integration and other chromatographic calculations were performed using CHROMELEON software (Thermo Scientific, USA). Identification and quantification were achieved via comparisons to standards (Sigma-Aldrich).

For extracellular metabolites, the supernatants of cultures were analyzed by HPLC. For the analysis of intracellular metabolites, mannitol was extracted from crushed lyophilized cells. In order to obtain comparable results, 100 mg of desiccated yeast extracts were suspended in 1000 μL of water and boiled for 10 min [33]. Mannitol was analyzed from supernatants by HPLC. Glycogen concentration was measured using a modified version of the protocol of Parrou and François [45]. 10 mg of desiccated yeast extracts were suspended in 250 μL of 0.25 M Na_2CO_3 and incubated 4 h at 95 °C. Then pH was adjusted adding 150 μL of 1 M acetic acid and 600 μL of 0.2 M of sodium acetate pH 5.2. Finally, glycogen was degraded into glucose by adding amyloglucosidase (Sigma-Aldrich) and incubating the suspension at 57 °C over night. Negative controls were obtained omitting amyloglucosidase. Glucose was analyzed from supernatants by HPLC and glycogen content was deduced from glucose content.

2.7. $NADP^+$/NADPH determination

$NADP^+$ and NADPH contents were determined from 5 mg of dry cells, using the NADP/NADPH Quantification Kit (Sigma-Aldrich), according to the manufacturer's instructions.

3. Results and discussion

3.1. FA production during growth on non-lipogenic and lipogenic medium

ATP-citrate lyase (Acl) and malic enzyme (Mae) are described as major enzymes for FA synthesis in oleaginous organisms. A strong increase of their activities has been reported in different studies during lipid accumulation in *T. cutaneum* and in *Y. lipolytica* [23,24]. To better analyze the role of Acl and Mae, we designed a non-lipogenic medium ($YNBD_2$) and a lipogenic medium (YNBCN30, presenting a C/N ratio of 30). Growth and FA production of JMY2900 (called reference strain in this paper) were compared in both media. Growth of the strain appears similar in both media (Fig. 2A), however, lipid accumulation was higher in the lipogenic medium (Fig. 2B). Indeed, FA accumulation reached 14.8% of cell dry weight (CDW) in lipogenic medium whereas it reached only 5.8% CDW in non-lipogenic medium (Fig. 2B), indicating a strong

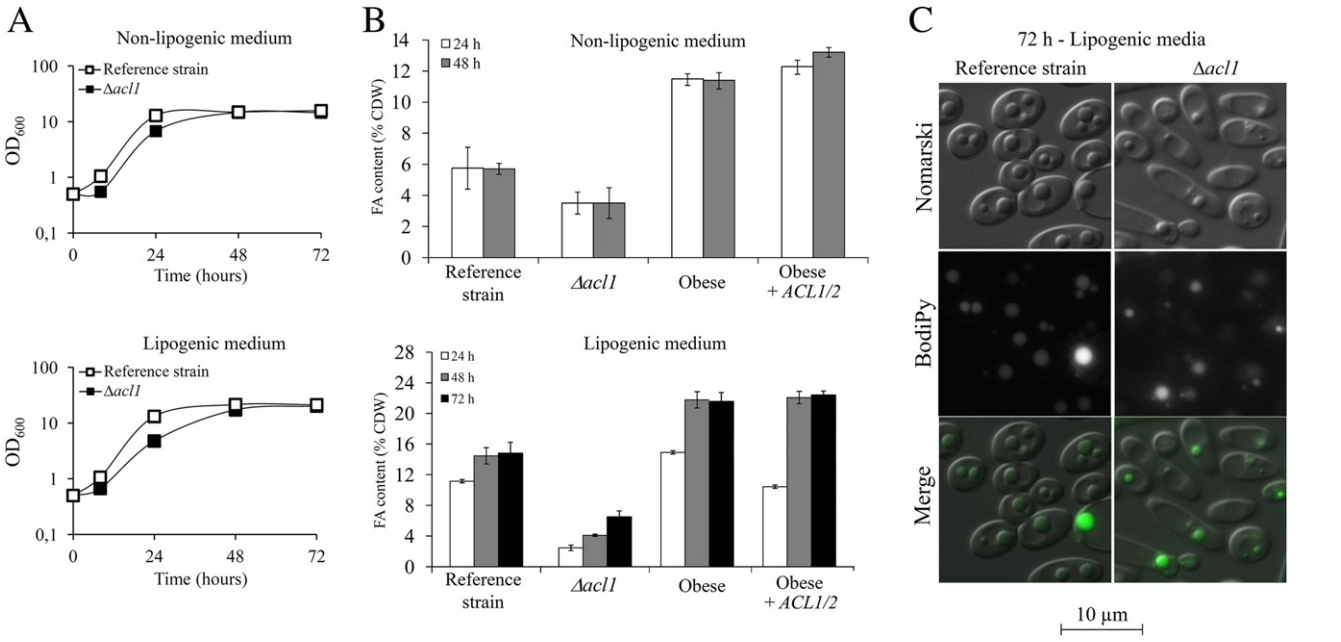

Fig. 2. Growth and FA content in various strains of *Y. lipolytica* inactivated for *ACL1* or overexpressing *ACL1* and *ACL2*. Strains were grown on lipogenic medium or non-lipogenic for 72 h. Growth (A) and FA content (B) were analyzed over the time. Results presented are mean values ± S.D. from three independent biological replicates. Panel C shows the modification of lipid bodies' number and size between the reference strain and the mutant strain inactivated for *ACL1*. A representative result is shown.

difference in their capacity to produce FA in these two media. These media should help us to decipher the role of Acl or Mae in *Y. lipolytica*.

3.2. Inactivation, but not overexpression, of ACL1 alters Y. lipolytica physiology and FA production

Acl is composed of two sub-units, Acl1 and Acl2, and Chen et al. [21] have shown that inactivation of *ACL1* in *A. niger* was sufficient to totally inhibit Acl activity. To examine the role of Acl in FA synthesis in *Y. lipolytica*, the *ACL1* gene was inactivated and FA content of JMY2215, the *Δacl1* mutant, was compared to reference strain. Cells were grown on non-lipogenic medium for 48 h and on lipogenic medium for 72 h. The growth of the *Δacl1* mutant is delayed on both media as compared to the reference strain, even though it reaches the same final OD_{600} as reference strain (Fig. 2A).

FA content was analyzed by gas chromatography after 24 and 48 h of growth on non-lipogenic medium and after 24, 48 and 72 h of growth on lipogenic medium. FA content in non-lipogenic medium reaches 5.8% of CDW in reference strain at 24 and 48 h of growth. In the *Δacl1* mutant, FA content is decreased by 40% at both time points (Fig. 2B). This result is surprising, because Acl has been proposed to be important mostly when cells are growing in lipogenic media, with a favorable C/N ratio. Indeed, when nitrogen is limited, AMP deaminase is activated leading to a decrease in mitochondrial AMP concentration, which inhibits isocitrate dehydrogenase (Fig. 1). This blocks the TCA cycle at the isocitrate level and citrate is accumulated in mitochondria. Finally, citrate is transferred to cytoplasm to be cleaved by Acl into acetyl-CoA and oxaloacetate (Fig. 1) [1,15]. Thus, our results show that even on a non-lipogenic medium, Acl participates in FA synthesis in *Y. lipolytica*.

In lipogenic medium, the inactivation of *ACL1* dramatically reduces FA content (Fig. 2B, C). At 24, 48 and 72 h of growth, FA represents 5.8, 14.5 and 14.8% of CDW of the reference strain, whereas it represents only 2.5, 5 and 6% of CDW in the *Δacl1* mutant (Fig. 2B). The *Δacl1* mutant produces a similar content of FA as the reference strain cultivated on non-lipogenic medium. The absence of functional Acl reduces FA content by 60 to 80% in lipogenic medium. Additionally a decrease in size and number of LBs in *Δacl1* compared to the reference strain is observed (Fig. 2C).

Moreover, the *ACL1* mutation not only affects lipid content, but also FA profile (Table 2). In the reference strain, after 24 and 48 h of growth on non-lipogenic medium or after 48 and 72 h of growth on lipogenic medium, the FA profile is composed of 8–10% of C16:0, 5% of $C16:1_{(n-7)}$, 2–5% of C18:0, 55–57% of $C18:1_{(n-9)}$ and 13–23% of $C18:2_{(n-6,9)}$. In the same conditions, *Δacl1* mutant presents more unsaturated FA. Indeed, it contains, 15–60% more $C16:1_{(n-7)}$, 115–280% more $C18:2_{(n-6,9),}$30–45% less C18:0, and 35–70% less $C18:1_{(n-9)}$ than reference strain. Increase of proportion of $C16:1_{(n-7)}$ may result from the increase of conversion of C16:0 into $C16:1_{(n-7)}$ and the decrease of $C18:1_{(n-9)}$ could be attributed to its conversion into $C18:2_{(n-6,9)}$. Lazar et al. [36] had previously observed that a *Y. lipolytica* strain improved for FA synthesis presents

Table 2
FA profile of *Δacl1* and reference strain grown on non-lipogenic or lipogenic medium.

	Non-lipogenic medium ($YNBD_2$)				Lipogenic medium (YNBCN30)					
	Reference strain		*Δacl1*		Reference strain			*Δacl1*		
	24 h	48 h	24 h	48 h	24 h	48 h	72 h	24 h	48 h	72 h
C16:0	8.9 ± 0.6	8.2 ± 0.2	17.0 ± 2	8.6 ± 0.2	10.9 ± 0.1	11.3 ± 0.8	10.5 ± 0.5	13.8 ± 0.8	10.3 ± 0.8	8.9 ± 0.5
C16:1(n−7)	5.6 ± 0.5	4.9 ± 0.2	8.9 ± 0.2	5.7 ± 0.1	4.6 ± 0.0	5.4 ± 0.3	5.4 ± 0.3	4.5 ± 0.2	7.4 ± 0.2	7 ± 0.1
C18:0	3.0 ± 0.2	3.0 ± 0.1	3.5 ± 0.1	2.0 ± 0.0	5.2 ± 0.2	5.7 ± 0.1	5.3 ± 0.2	5 ± 0.1	3.5 ± 1.0	2.9 ± 0.0
C18:1(n−9)	57.9 ± 0.4	58.0 ± 0.2	21.3 ± 3.1	32.4 ± 3.8	56.9 ± 0.2	56.1 ± 0.4	56.1 ± 1.0	18.2 ± 0.1	28.4 ± 1.5	36.8 ± 0.2
C18:2(n−6)	20.8 ± 1.6	21.2 ± 0.3	49.1 ± 4.5	45.6 ± 4.6	15.6 ± 0.4	13.2 ± 0.2	13.4 ± 0.0	58.5 ± 0.1	45.1 ± 0.8	35.2 ± 2.0
Total FA	96.1	95.2	99.8	94.3	93.1	91.7	90.7	100.0	94.8	90.9

Three independent experiments were done from separated series and cultures. Results are given as mean ± SD.

shorter and lower unsaturated FA. Finally, the capacity of cells to produce low or high level of FAs may influence unsaturation of FAs.

Finally, to better characterize the role of Acl in *Y. lipolytica*, *ACL1* and *ACL2* were overexpressed in PO1d (an auxotrophic normal strain). As already reported by Blazeck et al. [10], no improvement of lipid content was observed on non-lipogenic or lipogenic media (data not shown). Both genes were overexpressed in a *Y. lipolytica* strain optimized for FA accumulation (JMY3501, called in this paper "Obese"). This strain, previously described in Lazar et al. [36], is inactivated for TAG degradation (inactivation of the intracellular lipase Tgl4 [46]), β-oxidation (inactivation of the 6 acyl-CoA oxidases [47]) and overexpresses *GPD1* (involved in glycerol-3-phosphate synthesis [5]) and *DGA2* (one of the major acytransferases, involved in the last step of TAG synthesis [4]). This Obese strain produces 1.7- to 2-times more FA than the prototrophic reference strain [36]. Surprisingly, overexpression of *ACL1* and *ACL2* causes a slight improvement in FA content (+15%) on non-lipogenic medium but not on lipogenic medium (Fig. 2B).

All together, our results represent the first direct demonstration of the essential role of Acl into FA synthesis in oleaginous organisms. Interestingly, overexpression of *Y. lipolytica* Acl in the non-oleaginous fungus *Ashbya gossypii*, which does not possess *ACL* gene, improves by 33% the capacity of this fungus to produce lipids [48]. Moreover, Zhang et al. [49], have recently shown that overexpression of the mouse Acl, presenting a much lower K_m than the *Y. lipolytica* Acl, strongly improves FA synthesis in *Y. lipolytica*. Finally, if Acl from *Y. lipolytica* is important for FA synthesis, its low affinity for citrate may limit the capacity of *Y. lipolytica* to produce FA and may explain why production of lipids is generally counteracted or limited by citrate production.

3.3. Inactivation of ACL1 modifies carbon metabolic flux in Y. lipolytica

Δacl1 grows poorly and accumulates a low amount of lipids. However, as shown in Table 3, this strain is able to consume all glucose from the medium, but with a slower rate than the reference strain. After 24 h of growth on non-lipogenic medium, the reference strain has already consumed all (20 g/L) of the glucose, whereas 8.8 g/L are still present with *Δacl1*. In this mutant, glucose is totally consumed after 48 h of growth and is transiently converted into citrate (0.24 g/L) and mannitol (1.25 g/L) between 0 and 24 h. No citrate and no mannitol were detected in the reference strain. By 48 h these compounds have been reconsumed by the mutant strain.

After 24 h of growth on lipogenic medium, the reference strain has already consumed 90% of glucose, whereas *Δacl1* has only consumed 20%. Glucose totally disappears after 48 h of growth for reference strain and after 72 h of growth for *Δacl1*. However, although *Δacl1* grows and consumes glucose slower than the reference strain, it secretes more mannitol and citrate (Table 3). Indeed, after 48 and 72 h, 18% (w/w) of glucose has been converted into mannitol by *Δacl1*, whereas reference strain has converted 10% (w/w) of glucose into mannitol, which is quickly reconsumed after glucose exhaustion. Moreover, deletion of *ACL1* results in a briefly elevated production of citrate. After 48 h, 25% (w/w) of glucose has been converted into citrate, whereas citrate is only very weakly produced by reference strain. These last results confirm that metabolic flux in the *Δacl1* mutant is blocked at citrate level and is somehow redirected to mannitol. Interestingly, inactivation of *ACL1* in *A. niger* results in a decrease of citric acid production and in an increase of succinic acid production [20,21]. These data suggest that *ACL1* inactivation affects metabolic flux in various organisms differently.

3.4. Deletion of MAE1 induces FA synthesis in the optimized FA producing strain of Y. lipolytica but not in WT background

The positive role of Mae, by providing NADPH, on FA synthesis is now well accepted for various oleaginous microorganisms [12,25–30]. However, in *Y. lipolytica*, its role appears controversial, since this yeast does not possess cytosolic Mae, but only a mitochondrial form, which may not be involved in NADPH production [30]. In order to analyze if this enzyme may present a potential role in lipid metabolism, *MAE1* was inactivated. Finally, similar results to Blazeck et al. [10] were observed, i.e. the deletion of *MAE1* does not affect growth or lipid accumulation, in either non-lipogenic or lipogenic media, in a "normal" strain (Fig. 3A, B, C). JMY4162, the mutant strain inactivated for *Δmae1* (Table 1), and the mutant obtained by Blazeck et al. [10] are poor producers of FA. The very low level of FA in these strains may hide the phenotype of *Δmae1* mutant. We anticipate that, in a strain producing more FA the effect of *MAE1* inactivation may appear more clearly.

MAE1 was inactivated in Obese strain (JMY3501 [36]), generating the JMY4501 strain (called Obese *Δmae1*, Table 1). FA content of Obese *Δmae1* was compared to the ones of reference strain and Obese during growth on lipogenic medium. The three different strains present similar growth on this medium (Fig. 3A and Table 4). At 24 and 48 h of growth, Obese and Obese *Δmae1* present similar FA content (22 to 23% CDW). Surprisingly, at 72 h of growth, the FA content of Obese *Δmae1* reached 31.8% of CDW whereas in Obese, it stayed at 23.3% of CDW. This improvement of 35% of FA content was totally unexpected and clearly confirms that Mae in *Y. lipolytica* is absolutely not involved into FA synthesis and may even disfavor FA synthesis in some conditions.

3.5. Deletion of MAE1 induces remobilization of storage carbohydrates

The improvement of FA synthesis occurring between 48 and 72 h of growth in lipogenic medium for Obese *Δmae1* was surprising because glucose, the only carbon source present in this medium, has been entirely consumed (Table 4). To understand how this strain is still able to continue to synthesize FA after exhaustion of glucose, we analyzed by HPLC the extracellular or intracellular pools of mannitol, glycogen and organic acids during growth of reference strain, *Δmae1*, Obese and Obese *Δmae1*.

Table 3
Glucose consumption, production of FA (intracellular), biomass and extracellular citrate and mannitol during growth of *Δacl1* and reference strain grown on non-lipogenic or lipogenic medium.

	Non-lipogenic medium ($YNBD_2$)					Lipogenic medium (YNBCN30)						
Strain	0 h	Reference strain		*Δacl1*		0 h	Reference strain			*Δacl1*		
Time		24 h	48 h	24 h	48 h		24 h	48 h	72 h	24 h	48 h	72 h
Glucose (g/L)	20	n.d.	n.d.	8.8	n.d.	30	2.6	n.d.	n.d.	24.2	5.9	n.d.
Mannitol (g/L)	n.d.	n.d.	n.d.	1.3	n.d.	n.d.	2.5	n.d.	n.d.	0.7	4.4	5.3
Citrate (g/L)	n.d.	n.d.	n.d.	0.2	n.d.	n.d.	0.3	n.d.	n.d.	0.3	6.0	n.d.
FA (g/L)	/	0.26	0.50	0.08	0.26	/	0.75	1.48	1.75	0.06	0.36	0.69
Biomass (CDW g/L)	/	4.5	8.7	2.4	7.4	/	6.8	9.9	11.3	2	7.5	9.8

n.d., not detected; /, not determined.
Values are means from two to tree independent experiments. The standard deviations were ≤10% of the values shown.

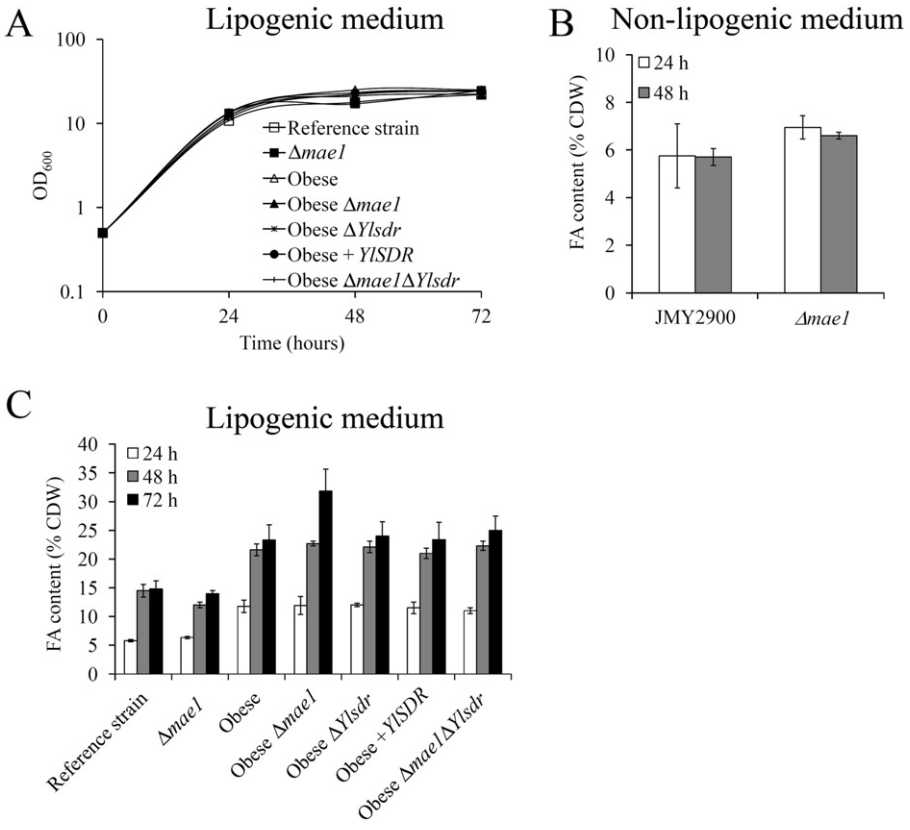

Fig. 3. Growth and FA content in various strains of *Y. lipolytica* inactivated for *MAE1*. Strains were grown on lipogenic medium or non-lipogenic for 72 h. Growth (A) and FA content (B, C) were analyzed. Results presented are mean values ± S.D. from three independent biological replicates.

Main metabolite differences were observed during the last 24 h of culture on lipogenic medium (between 48 to 72 h of growth), between Obese and Obese *Δmae1* (Table 4). These differences were observed only with analysis of intracellular metabolites. Indeed, glycogen content is entirely consumed by Obese *Δmae1* between 48 and 72 h. On the other hand, intracellular mannitol content is dramatically reduced (by 5 times) in Obese *Δmae1*. These results suggest that remobilization of storage carbohydrates (mannitol and glycogen) at the end of growth (between 48 and 72 h) may contribute to FA synthesis in Obese *Δmae1*. As previously observed with *Δacl1* mutant, there exists a negative correlation between the levels of FA and mannitol.

Our results confirm that Mae1 is not involved into FA synthesis in *Y. lipolytica* but, in some conditions, its inactivation may affect cellular management of pool of stored carbon. As previously suggested by Zhang et al. [30], Mae may not be a provider of NADPH for FA synthesis in *Y. lipolytica*. This raises the question of the supply of NADPH for FA synthesis in this yeast. In a recent review, C. Ratledge has proposed an alternative route for NADPH generation via a cytosolic isocitrate dehydrogenase ($NADP^+$-dependent) and Wasylenko et al. have demonstrated recently the importance of pentose phosphate pathway as NADPH provider for FA synthesis pathway [25,31]. Mannitol cycle (Fig. 4) may supply reducing power to the cell [32]. The balance found between mannitol and FA contents in *Δacl1* or in Obese *Δmae1*, may suggest that mannitol cycle plays a role in the supply of NADPH for FA synthesis in *Y. lipolytica* (Fig. 4). However, the correlation between mannitol and FA may also be explained by a system of communicating vessels between two carbon storage pools.

3.6. Mannitol metabolism in Y. lipolytica

In *Ascomycetes*, mannitol metabolism has been well established and takes place through the mannitol cycle (Fig. 4) [32,50]. A link between mannitol cycle and lipid synthesis was previously described in the non oleaginous fungus *Alternaria alternata* [32]. Indeed, these authors have demonstrated that NADPH produced by mannitol cycle in *A. alternata* is enough to supply all lipid synthesis of the cells. Moreover, they observed that a strain having low lipid content, presented high mannitol content. This result suggests that cells inhibited in lipid synthesis may reduce mannitol degradation to avoid NADPH overproduction. It may be the same in the case of *Δacl1* mutant. We analyzed NADPH level in reference strain and in *Δacl1* mutant (Table S2). $NADP^+$ and NADPH levels between the two strains appear to be relatively similar. In fact, *Δacl1* presents a delay of 24 h compared with reference strain. This delay in values can be attributed to differences of growth between two strains. Moreover, Wasylenko et al. (2015) demonstrated the essential role of pentose phosphate pathway as NADPH provider for FA synthesis [31]. All together, these data suggest that mannitol metabolism and FA are not connected at the NADPH level. In *Botrytis cinerea*, inactivation of mannitol pathways redirects metabolic flux to other carbon storage molecules, trehalose and glycogen [33]. In *Y. lipolytica*, the results we obtained may be explained similarly, i.e. a defect of FA synthesis may redirect carbon flux to mannitol synthesis and vice versa.

Genes and enzymes involved in mannitol metabolism are not well described in *Ascomycetes* yeasts and according to that, in *Y. lipolytica*. Recently, Napora et al. [51] purified and biochemically characterized an enzyme encoded by *YALI0B16192g* (named *YlSDR*) which presents characteristics of a NADP(H)-dependent Mdh. By BLAST analysis, we were able to identify 2 other genes encoding potential mannitol dehydrogenases, *YALI0D18964g* and *YALI0E12463g*. *YALI0B16192g* and *YALI0D18964g* share 83% identity and have 51% and 49% identities with the *B. cinerea* NADPH-dependent mannitol-2-dehydrogenase, BcMdh (Table S3) [33]. On the other hand, *YALI0E12463g* may encode for a very distant Mdh, sharing 28% identity with the *Tuber borchii* NADPH-dependent mannitol-2-dehydrogenase TbMdh (Table S3). Actually, TbMdh is very distant from the classical Mdh and belongs to a new subfamily of Mdh (Table S3) [52]. No genes encoding potential

Table 4
Biomass and metabolites produced by various strains of *Y. lipolytica* grown 24 h, 48 h or 72 h on lipogenic medium.

	Biomass (CDW g/L)															
		Reference strain			*Δmae1*			Obese			Obese *Δmae1*			Obese *Δmae1ΔYlsdr*		
	0 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Biomass	/	6.8 ± 0.4	9.9 ± 0.4	11.3 ± 0.1	7.9 ± 0.6	10.3 ± 0.6	10.5 ± 0.1	7.1 ± 0.1	10.4 ± 0.2	10.5 ± 0.4	5.9 ± 0.2	9.0 ± 0.1	11.0 ± 0.1	7.2 ± 0.2	10.8 ± 0.5	10.2 ± 0.2
	Extracellular metabolites (g/L)															
		Reference strain			*Δmae1*			Obese			Obese *Δmae1*			Obese*Δmae1ΔYlsdr*		
	0 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Glucose	30.00	12.2 ± 0.1	0.9 ± 0.2	n.d.	13.6 ± 0.8	2.6 ± 0.7	n.d.	15.0 ± 0.4	1.5 ± 0.5	n.d.	15.6 ± 0.1	n.d.	n.d.	14.3 ± 0.2	n.d.	n.d.
Mannitol	n.d.	0.5 ± 0.0	1.3 ± 0.1	n.d.	0.5 ± 0.0	1.6 ± 0.2	0.4 ± 0.6	0.3 ± 0.0	0.8 ± 0.0	n.d.	0.3 ± 0.1	0.4 ± 0.2	n.d.	0.4 ± 0.1	0.6 ± 0.2	n.d.
Citrate	n.d.	0.7 ± 0.0	1.4 ± 0.0	1.1 ± 0.1	0.9 ± 0.0	1.8 ± 0.8	0.8 ± 0.1	0.4 ± 0.0	2.2 ± 0.1	n.d.	0.5 ± 0.1	1.3 ± 0.3	0.2 ± 0.3	0.4 ± 0.1	1.0 ± 0.2	0.1 ± 0.1
Malate	n.d.	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.00	0.1 ± 0.0	1.4 ± 0.1	n.d.	n.d.	n.d.	1.4 ± 0.0	n.d.	0.5 ± 0.2	1.1 ± 0.2
	Intracellular metabolites (mg/CDW g)															
		Reference strain			*Δmae1*			Obese			Obese *Δmae1*			Obese*Δmae1ΔYlsdr*		
	0 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Glycogen	/	91.6 ± 12.7	69.1 ± 2.1	47.6 ± 3.1	81.9 ± 0.0	58.9 ± 11.3	44.2 ± 4.0	83.8 ± 0.6	55.9 ± 0.7	27.1 ± 0.2	59.7 ± 0.9	**22.6 ± 6.1**	**n.d.**	81.8 ± 10.2	47.7 ± 1.4	19.3 ± 0.8
Mannitol	/	59.2 ± 1.7	52.1 ± 0.5	66.7 ± 0.2	57.4 ± 9.5	54.2 ± 6.5	71.5 ± 4.0	40.1 ± 3.6	44.5 ± 0.2	30.5 ± 4.0	40.0 ± 10.4	47.1 ± 0.4	**6.6 ± 2.3**	51.6 ± 0.0	62.5 ± 0.0	50.4 ± 0.3
FA	/	58.0 ± 2.0	145.0 ± 11.0	148.0 ± 14.0	63.5 ± 2.0	120.0 ± 5.0	140.0 ± 5.0	117.7 ± 11.0	216.0 ± 10.4	233.3 ± 26.2	119.0 ± 15.6	227.0 ± 4.2	**318.5 ± 38.0**	110.0 ± 5.0	223.0 ± 8.0	250.0 ± 25.0

n.d., not detected; /, not determined.
Three independent experiments were done from separated series and cultures. Results are given as mean ± SD.

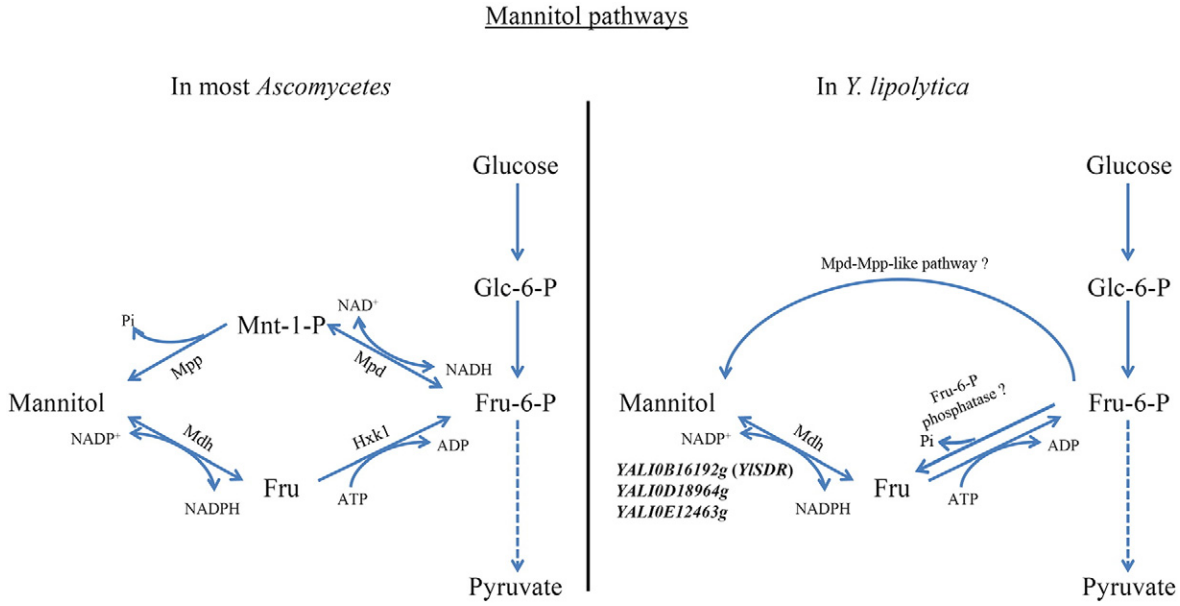

Fig. 4. Model of mannitol pathways in most oleaginous fungi and in *Y. lipolytica*. The current admitted mannitol pathway in most *Ascomycetes* (left panel) and the *Y. lipolytica* one (right panel) where homologues for Mdh and Hxk1 were identified while it lacks Mpd and Mpp. Glc-6-P, Glucose-6-phosphate; Fru-6-P, Fructose-6-phosphate; Mnt-1-P, Mannitol-1-phosphate; Fru, Fructose; Hxk1, Hexokinase; Mdh, Mannitol dehydrogenase; Mpd, Mannitol-1-phosphate dehydrogenase; Mpp, Mannitol-1-phosphatase.

mannitol-1-phosphate dehydrogenase or mannitol-1-phosphatase have been found in the *Y. lipolytica* genome, which may suggest that contrary to most of *Ascomycetes* fungi, mannitol synthesis and degradation occur exclusively by the Mdh pathway (Fig. 4). However, because the *Δacl1* mutant is able to produce mannitol from glucose and because the WT strain of *Y. lipolytica* can produce mannitol from

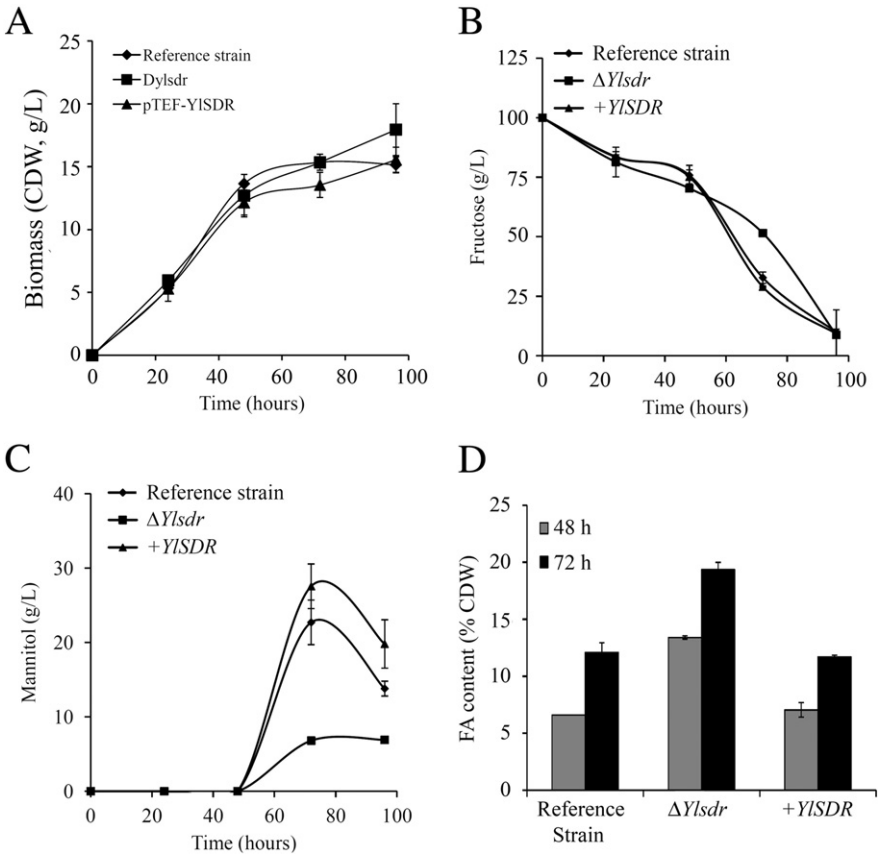

Fig. 5. Growth, consumption of fructose and production of mannitol and FA by *Y. lipolytica* strains inactivated for- or overexpressing *YlSDR*. Reference strain, *ΔYlsdr* and *+YlSDR* were grown on Mfru10% for 96 h. Consumption of fructose and production of mannitol and FA were analyzed over the time. Graphics A represents the biomass (CDW, g/L) of the different strains at the different time points of the kinetic. Graphics B represents the consumption of fructose during growth on MFru10% over 96 h. Graphics C represents the production of secreted mannitol during growth on MFru10% over 96 h. Graphics D represents the FA content (% of CDW) during growth on MFru10% after 48 and 72 h of growth. All the results presented are mean values ± S.D. from three independent biological replicates.

glycerol [34], a pathway converting Fru-6-P into mannitol may exist in *Y. lipolytica*.

3.7. Inactivation and overexpression of YlSDR of Y. lipolytica modify mannitol production and FA content on fructose medium

Mannitol overproduction in the *Δacl1* mutant may result from a redirection of carbon metabolic flux from FA to mannitol. On the other hand, mannitol remobilization may participate to FA improvement in Obese *Δmae1*. Relationship between mannitol and FA synthesis could be easily analyzed comparing FA accumulation of cells grown on mannitol versus glucose. However, *Y. lipolytica* is unable to grow on mannitol (data not shown). Finally, to validate the relationship between mannitol and FA in *Y. lipolytica*, we constructed strains inactivated or overexpressing the gene *YlSDR*, encoding the putative NADP(H)-dependent mannitol dehydrogenase, generating JMY4381 (called *ΔYlsdr*) and JMY4812 (called +*YlSDR*) strains, respectively. The resulting strains were grown on media known to favor mannitol production in *Y. lipolytica*, containing either 10% glycerol or 10% fructose (Juszczyk P. personal communication). Growth, glycerol and fructose concentrations in the medium, FA content and mannitol production were quantified for both strains and compared to the reference strain JMY2900 (Fig. 5 and Fig. S1 and data not shown).

Strains present similar growth on both media (Fig. 5A and data not shown) and similar kinetics of glycerol or fructose consumption (Fig. 5B and Fig. S1A). No differences in mannitol and FA production were observed on glycerol (Fig. S1B and data not shown). Striking results were obtained during growth on fructose. Indeed, inactivation of *YlSDR* drastically reduced the capacity of the mutant strain to produce mannitol from fructose (−70%, Fig. 5C) without affecting its growth (Fig. 5A). On the other hand, overexpression of *YlSDR* slightly improved mannitol production from fructose (+18%, Fig. 5C). At 72 h of growth, a maximal level of mannitol was reached by the three strains. Reference strain produced up to 23 g/L of mannitol, +*YlSDR* produced up to 27 g/L whereas *ΔYlsdr* produced only up to 6.8 g/L of mannitol. Moreover, mannitol is reused by the reference strain and +*YlSDR* between 72 h and 96 h but not by *ΔYlsdr* strain. These results indicate that *ΔYlsdr* mutant is strongly impaired for mannitol production from fructose, but not from glycerol (Fig. 5C and Fig. S1B) or glucose (data not shown), confirming that *YlSDR* really encodes a mannitol dehydrogenase, as already suggested by Napora et al. [51]. Moreover *YlSDR* is not only important for mannitol synthesis but also for mannitol consumption. However, the *ΔYlsdr* mutant is still able to produce mannitol from fructose, suggesting that other enzymes, maybe encoded by *YALI0D18964g* and *YALI0E12463g*, are involved in conversion of fructose to mannitol. Moreover, inactivation of *YlSDR* did not alter mannitol synthesis on glycerol and glucose (Fig. S1B and data not shown), confirming that other enzymes or pathways are required to produce mannitol from glycerol or glucose through Fru-6-P, suggesting the existence of a Mpd–Mpp-like pathway or of a Fru-6-P phosphatase (Fig. 4).

Fructose appears to be the best substrate to observe differences of mannitol production in strains altered for *YlSDR*. FA content was measured in cells grown on this sugar for 48 h and 72 h (Fig. 5D). The reference strain and +*YlSDR* present a similar FA content of 6 to 7% and 12% of CDW at 48 and 72 h of culture, respectively. It is possible that the slight increase in mannitol production observed in the +*YlSDR* strain is not enough to alter FA synthesis. However, until 72 h, it may be sufficient responsible for the slight decrease of growth of this strain (Fig. 5A). On the other hand, inactivation of *YlSDR* strongly increases FA synthesis (Fig. 5D). Indeed, FA content is increased by 100% and 60% at 48 h and 72 h of growth, demonstrating clearly that level of mannitol and of FA are intimately linked in *Y. lipolytica* during growth on fructose.

As shown previously, overaccumulation of FA in Obese *Δmae1* compared to Obese strain was associated with a remobilization of intracellular mannitol. To confirm that mannitol remobilization was really involved in FA synthesis and to decipher the potential implication of YlSdr in this process, Obese *Δmae1* was inactivated for *YlSDR*. Intracellular mannitol and FA contents of the corresponding mutant (called Obese *Δmae1ΔYlsdr*) were compared to the one of Obese and Obese *Δmae1* (Table 4 and Fig. 3C). The inactivation of *YlSDR* in Obese *Δmae1* decreases the level of FA content to the level of Obese strain (Fig. 3C and Table 4). Moreover, inactivation of *YlSDR* in Obese *Δmae1* impaired remobilization of intracellular mannitol (Table 4). On the other hand, inactivation or overexpression of *YlSDR* in Obese strain does not modify FA content (Fig. 3C). These results clearly show that YlSdr mediates mannitol remobilization in Obese *Δmae1* and participates in FA synthesis improvement. Finally, our results altogether show that YlSdr is able to work in both directions, synthesis of mannitol during growth on fructose, and degradation of mannitol during various cellular processes as FA synthesis.

4. Conclusions and perspectives

The aim of this work was to understand the role of the Acl and the mitochondrial NAD(H)-dependent Mae in *Y. lipolytica*. The results obtained in this study clearly demonstrate that Acl is essential for FA synthesis in *Y. lipolytica*. Inactivation of *ACL1* dramatically reduces FA synthesis while promoting citrate and mannitol synthesis. On the other hand, results obtained with strains inactivated for *MAE1* definitively demonstrate that Mae is not involved in FA synthesis in *Y. lipolytica* but plays, in one way or another, a role in the management of storage carbon.

The most striking result was to discover the balance between mannitol and FA in strains inactivated for *ACL1* and *MAE1* mutants. The relationship between FA and mannitol was also observed when *YlSDR* was inactivated. On fructose, *ΔYlsdr* was strongly impaired for mannitol production and carbon flux was partially redirected to the FA synthesis pathway. The origin of the balance between mannitol and FA could be attributed to a competition for carbon flux. Additionally, the phenotype of the *ΔYlsdr* confirms that *YlSDR* encodes a mannitol dehydrogenase and represents the first example of this type of enzyme in *Ascomycetes* yeasts. Moreover, the *ΔYlsdr* mutant is still able to produce mannitol from fructose, probably due to the activity of enzymes encoded by *YALI0D18964g* and *YALI0E12463g*. The deletion of these genes, could help us to better understand mannitol metabolism in *Ascomycetes* yeasts.

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Competing interests

The authors declare that they have no competing interests.

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